comparing the light emission data from the cell in the presence of the agent to the light emission data from the cell in the absence of the agent, wherein a difference in light emission data is indicative of an effect on cell proliferation.

63. A method of screening mammalian cells to determine their susceptibility to treatment with an agent, comprising:

contacting cells containing a *Renilla* luciferase polypeptide or a polynucleotide encoding a *Renilla* luciferase with an agent; and

measuring light emissions from the cells in the presence and absence of the agent, wherein a difference in light emissions is indicative of the cells' susceptibility to treatment with the agent.

REMARKS

A. Regarding the Amendments

After entry of the amendments, claims 1 to 48 and 63 to 68 will be pending . The specific and claims 1, 18, 31 and 63 have been amended as set forth in the attached "Version With Markings To Show Changes Made." As amended, the claims are supported by the specification and the original claims. Applicant submits that the amendments to the claims are for clarity and should not be construed as amendments affecting patentability under *Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co.*, 234 F.3d 558, 56 USPQ2d 1865 (Fed. Cir. 2000) (en banc).

B. Objection to the Disclosure due to Informalities

The disclosure of the present application was objected to due to the following informalities, as set forth in Paper No. 7: the ATCC number on page 14, line 12 was omitted and the term "coelentrazine" was misspelled on page 20, line 2. It is respectfully submitted that the ATCC number is presently not included in the specification of the present application because

the sequence has not yet been submitted to the ATCC. Under 37 C.F.R. 1.809(b)(1), a deposit is required on or before the date of payment of the issue fee. Applicants therefore defer deposit and, subsequently, insertion of an ATCC number in the specification. The deposit will be made and the number will be inserted on or before payment of the issue fee.

With respect to the misspelling of the term "coelentrazine" on page 20, line 2, it is respectfully submitted that the amendment to the specification set forth above remedies this informality. It is therefore respectfully requested that the objection to the specification be withdrawn.

C. Rejections Under 35 U.S.C. §112, first paragraph

Claims 1 to 30 and 63 to 68 are rejected as allegedly not enabling under 35 U.S.C. §112, first paragraph. The rejection is respectfully traversed.

Initially it is alleged that the guidance and exemplification in the specification is insufficient to enable one of skill in the art to practice the claimed invention. Applicants respectfully disagree. 35 U.S.C. § 112, first paragraph, requires that one of skill in the art would have known, at the time of filing of the application, using the teachings of the application, how to practice the claimed invention. Applicants allege that the specification, as filed, would have enabled one of skill in the art to practice the claimed invention.

It is asserted in Paper No. 7 that because previous references have stated that coelenterazine was toxic to mammalian liver cells, that one of skill in the art would be unable to practice the claimed invention. The rejection is based upon the belief that the toxicity of coelenterazine and the effects of the agent on cell proliferation would be indistinguishable. Applicants respectfully disagree. In the Examples section of the application, on pages 25-26, there is an Example entitled "Assay for Luciferase Activity." In the example, Applicants show that coelenterazine is not included during cell culture. The coelenterazine is added to the cells and light emission is collected for fifteen seconds "immediately upon the addition of the

substrate." Clearly the cells will not grow in only fifteen seconds. Despite any concern regarding the toxicity of coelenterazine, one of skill in the art would be able to practice the present invention without undue experimentation by following the teachings in the specification of the present invention. Additionally, coelenterazine is not widely known in the art as toxic. In fact, those of skill in the art actually study its antioxidant properties (Biochem Pharmacol 2000 Aug 15;60(4):471-8, attached as Exhibit A). It would therefore be apparent to one of skill in the art that any effect seen on cell proliferation following the teaching of the present invention would be due to the test agent. Any concern regarding any potential detrimental effect of coelenterazine on cell proliferation due to prior disclosures is unfounded.

Additionally, Paper No. 7, states that "the specification is silent as to how valid light emission data can be collected in practicing the claimed methods." (Paper No. 7, page 4, second paragraph.) Applicants respectfully disagree. Methods of collection of light emission data are well known in the art. (See, for example, U.S. Pat. Nos. 5,418,155 and 5,22,658 which discuss collection of light emission data from *Renilla luciferase*. See, specifically, the example titled "Assay for luciferase activity"). Therefore, one of skill in the art would have known how to practice the claimed invention, using known methods of light collection. As such, one of skill in the art would be able to practice the present invention without undue experimentation.

The use of the invention in a human and the potential toxicity of coelenterazine is also discussed in Paper No. 7, on pages 4 to 5. This discussion does not appear to be a rejection or objection to the specification. With respect to this discussion, the Examiner's attention is respectfully drawn to the fact that the present invention is not intended for therapeutic use, but as a screening method. Therefore, any concern regarding the use of coelenterazine in humans is obviated. To further clarify this point, the independent claims of the present invention have been amended to claim "[a]n *in vitro* method." Therefore, any potential toxicity of coelenterazine in a human is not relevant to the claimed invention.

It is also stated in Paper No. 7 that the combined effects of coelenterazine and other agents may result in a greater level of cytotoxicity. It is respectfully submitted that, as discussed above, the toxicity of coelenterazine, even if amplified by an additional agent, is not relevant, as that toxicity would not affect proliferation. However, to clarify the relationship between a change in light emission and a change in cell proliferation, claim 18 has been amended to read "wherein a change in light emission is indicative of a corresponding change in cell proliferation." This amendment also addresses the statement in Paper No. 7 that the previous wording was inaccurate. It is respectfully submitted that, as amended, claim 18 accurately claims the relationship between a change in light emission and a change in cell proliferation.

Similarly, it is discussed in Paper No. 7 that the claimed invention has an "unacceptable degree of unpredictability" and therefore would require one skilled in the art to perform undue experimentation in performing the present invention. Applicants respectfully disagree. The references cited to show the "unacceptable degree of unpredictability" are from 1990 and 1998. The present application was filed in April, 2000. If, at the time of the references cited, the present invention had been predictable, the invention would not now fulfill the requirements for patentability. It is expected that the present invention would not have been obvious or predictable as of 1998. The Cree reference, as cited in Paper No. 7, states: "It is *unlikely* that molecular methods will fare much better." (Emphasis added.) However, it does not preclude the use of the present molecular methods. The present invention teaches that a correlation exists between luminescence and determination of cell proliferation in that a measurement of luminescence will allow a determination in change of cell number. (Specification, page 20, line 26 to page 21, line 9.) The correlation is used to determine the effect of an agent on cell proliferation, determine the cell proliferation of a cell or population of cells, or to screen mammalian cells to determine their susceptibility to treatment with an agent. Therefore, one of skill in the art practicing the present invention would not have to engage in undue experimentation.

The Office Action also asserts that the specification allegedly does not exemplify the claimed method for screening mammalian cells to determine their susceptibility to treatment with an agent. It is also stated that there are allegedly no working examples that teach the claimed method and that the examples do not set forth a method for determining prokaryotic cell proliferation. Applicants respectfully traverse the rejection.

Throughout the specification and in the Examples section, Applicants disclose that treatment with an agent is related to cell proliferation. It is respectfully submitted that it is has been shown in the art that various mammalian cells may be screened in order to determine their susceptibility to treatment with a cancer agent. Exhibit B sets forth the abstracts of four illustrative references that show this relationship. The Haug abstract, describes the finding that hydroxyurea, cytochalasin B, vinblastine, Razoxane and interferon all inhibit growth of murine fibrosarcoma cells. (Cell. Prolif. 1993 May; 26(3):251-61, attached hereto as Exhibit B.) Similarly, in the Malmberg abstract, the slow-down or arrest of growth of human ileocaecal adenocarcinoma cells, after exposure to 5-fluoro-2'-deoxyuridine is described. (Cell. Prolif. 1993 May; 26(3):291-303, attached hereto as Exhibit B.) The Shin reference shows the effects of a combination of 13-cRA, IFN-alpha and alpha-tocopherol on prevention of tumor recurrence. (J. Clin. Oncol. 2001 Jun 15; 19(12):3010-7, attached hereto as Exhibit B.) Finally, the Komatsu reference describes the inhibition of four out of five human tumor cell lines implanted into nude mice, by the agent cyclic hydroxamic-acid-containing peptide 31 (CHAP31). (Cancer Res. 2001 Jun 1; 61(11):4459-66, attached hereto as Exhibit B.) Similarly, the present invention exemplifies the effect of an agent on cellular proliferation and it is respectfully submitted that the relationship between cellular proliferation and susceptibility to treatment with an agent is well known in the art. Accordingly, Applicants respectfully request that the present rejection be withdrawn.

D. Rejections Under 35 U.S.C. §112, second paragraph

Claims 1 to 47 and 63 to 68 are rejected as allegedly incomplete under 35 U.S.C. §112, second paragraph, for omitting essential steps. The rejection is respectfully traversed.

Applicants respectfully submit that the claims do not omit essential steps. The steps allegedly omitted are: 1) a cellular lysate is prepared and 2) coelenterazine is added to the lysate and 3) light emission data is collected from cells in the presence and absence of an agent. Applicants respectfully submit that these steps are not required for the practice of the present invention. As coelenterazine is hydrophobic, it may penetrate the cells without the assistance of a detergent to make the cell membrane permeable.

Additional methods of screening cells are available to one of skill in the art besides lysing the cells. These other methods might include the use of the tetrazoleum component MTT or use of Trypan Blue. Both would allow assays of cellular proliferation without lysing the cells. Because other methods of screening cells are available and are known to those of skill in the art, Applicants respectfully submit that no steps have been omitted from claims 1-17, 18-30, 31-47 or 63-68. It is therefore requested that the rejection be removed.

Additionally, claims 1-47 and 63-68 are rejected as indefinite under 35 U.S.C. §112, second paragraph. This rejection is respectfully traversed.

The rejection of claims 1-17 alleges that the phrase "an agent" in line 3 of claim 1 is indefinite. This allegation is based on the fact that the term "an agent" is used in line 1 of the claim and therefore it is allegedly indefinite whether the use of the term in line 3 refers to the same agent as in line 1. The use of the term "an agent" in line 1 is part of the preamble of the claim. Generally, a preamble is not considered limiting to a claim unless it breathes life and meaning into the claim. (See MPEP 2111.02.) In the present invention, the claim following the transitional phrase "comprising" stands alone. It is therefore unnecessary for the term "an agent" in line 1 to serve as an antecedent basis for the use of "an agent" in line 3, or for the term "agent"

in line 3 to be preceded by a definite article. As such, use of the indefinite article "an" is proper in line 3.

The rejection of claims 18-30, claims 31-47 and claims 63-68 is similar to the rejection of claims 1-17. All are alleged to be indefinite for the use of an indefinite article in line 3. As discussed above, the use of the indefinite article in each of claims 18, 31, and 63 is acceptable, as it is a portion of the claim that stands alone.

As the use of indefinite terms in claims 1, 18, 31, and 63 in line 3 of each claim do not require an antecedent basis, as used, it is submitted that those claims are not indefinite under 35 U.S.C. §112. Accordingly, the removal of the rejection of claims 1-47 and 63-68 is respectfully requested.

Claims 63 to 68 are also rejected as indefinite under 35 U.C.C. §112, second paragraph, as the relationship between cell proliferation and susceptibility to treatment is unclear. Claim 63 has been amended to include the phrase "wherein a difference in light emissions is indicative of the cells' susceptibility to treatment with the agent." As such, it is respectfully requested that the rejection be removed.

E. Rejections Under 35 U.S.C. §103

Claims 1 to 47 and 63 to 68 are rejected under 35 U.S.C. §103(a) as allegedly unpatentable over Cree in view of Virta, et al, Edinger, et al, Prosser, et al, and further in view of U.S. Pat. Nos. 5,292,658 A and 6,171,809 B1. The rejection is respectfully traversed.

None of the references cited as allegedly rendering the present invention obvious teach or suggest that the effect of an agent on cell proliferation may be tested via measurement of light emission using *Renilla* luciferase. Specifically, the Cree reference teaches determination of cell viability based on luminescence. It is ATP based. It does not teach or suggest the use of luminescence using *Renilla* luciferase.

Similarly, the Virta reference discusses the connection between prokaryotic cell viability and light emission, but uses a known agent to establish this connection. Virta contains no teaching or suggestion to use the process in reverse, using the light emission of *Renilla* luciferase to determine the effect of an agent of unknown properties on proliferation.

The Edinger reference also does not teach or suggest the use of *Renilla* luciferase to determine the effect of an agent on cellular proliferation. Accordingly, this reference does not teach or suggest the present invention.

The teachings of the Prosser reference and U.S. Pat. Nos. 5,292,658 A and 6,171,809 B1 cannot overcome the deficiencies of the Cree, Virta and Edinger references. None of the cited references, alone or in combination teach or suggest the use of *Renilla* luciferase to determine the effect of an agent on cellular proliferation.

Cree, et al., Virta, et al, Edinger, et al, Prosser, et al, and U.S. Pat. Nos. 5,292,658 A and 6,171,809 B1 are provided as discussed above. None of the cited references, alone or in combination, teach or suggest all of the claimed aspects to the present invention. Accordingly, it is respectfully requested that this rejection of the claims under 35 U.S.C. § 103, be removed.

CONCLUSION

In Summary, for the reasons set forth herein, Applicants maintain that claims 1 to 48 and 63 to 68 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

No fee is deemed necessary in connection with the filing of this response. However, if any fee is deemed necessary, the Commissioner is authorized to charge (or apply any credits to) Deposit Account No.: 50-1355. The Examiner is invited to contact Applicant's undersigned representative if there are any questions related to this matter.

Respectfully submitted,

Date: September 7, 2001

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION

The original paragraph on page 19, line 27 to page 20, line 13 has been amended as follows:

The cells containing a *Renilla* luciferase are cultured under conditions that allow expression of the *Renilla* luciferase. The luciferase activity can then be measured *in vivo* or *in vitro* (see, for example, Lorenzo *et al.*, J Biolumin Chemilumin, 11(1):31-7, 1996, which is incorporated by reference herein) by providing the cell culture with the substrate [coelentrazine] coelenterazine. Typically the [coelentrazine] coelenterazine will be in an amount of about 0.05 μM to about 5 μM, depending, for example, upon the assay conditions (e.g., whole cell, lysate, purified protein). Alternatively, the cells can be lysed prior to addition of the substrate. In such instances the cells can be lysed by adding appropriate buffer or by mechanical disruption or other methods known to those of skill in this art. The vessels, particularly the microtiter plates, can be placed in commercially available instruments for measuring light, such as a plate reader, which can be interfaced with a computer for data analysis. Depending upon the assay type, one skilled in the art can develop various methods to determine a change in cell number. For example, where cell death is measured, the cells can be washed between measurements to determine the number of cells or luciferase activity present before and after the wash. For example, a decrease in the number of cells over a period of time is indicative of cell death.

IN THE CLAIMS

1. (Amended) [A] An *in vitro* method for determining the effect of an agent on cell proliferation, comprising:

contacting a cell containing a *Renilla* luciferase polypeptide or a polynucleotide encoding a *Renilla* luciferase with an agent suspected of modulating cell proliferation under conditions that allow the agent and the cell to interact; and

comparing the light emission data from the cell in the presence of the agent to the light emission data from the cell in the absence of the agent, wherein a difference in light emission data is indicative of an effect on cell proliferation.

18. (Amended) [A] An *in vitro* method for determining cell proliferation of a cell or population of cells comprising:

obtaining light emission data from a cell containing a *Renilla* luciferase polypeptide or a polynucleotide encoding a *Renilla* luciferase over a period of time wherein a change in light emission data is indicative of a corresponding change in cell proliferation.

31. (Amended) [A] An *in vitro* method for determining the effect of an agent on cell proliferation, the method comprising:

transfecting a cell obtained from a sample with a vector containing a polynucleotide sequence encoding a *Renilla* luciferase;

contacting the transfected cell with an agent suspected of modulating cell proliferation under conditions that allow the agent and the cell to interact; and

comparing the light emission data from the cell in the presence of the agent to the light emission data from the cell in the absence of the agent, wherein a difference in light emission data is indicative of an effect on cell proliferation.

63. (Amended) A method of screening mammalian cells to determine their susceptibility to treatment with an agent, comprising:

contacting cells containing a *Renilla* luciferase polypeptide or a polynucleotide encoding a *Renilla* luciferase with an agent; and

measuring light emissions from the cells in the presence and absence of the agent, wherein a difference in light emissions is indicative of [an agent which affects cell proliferation] the cells' susceptibility to treatment with the agent.

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Expression of the *Renilla reniformis* Luciferase Gene in Mammalian Cells

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A cDNA encoding the *Renilla reniformis* luciferase was expressed in simian and murine cells in a transient and stable manner, respectively. Light emission catalyzed by luciferase was detected from transfected cells both *in vitro* and *in vivo*. This work establishes the *Renilla* luciferase gene as a new efficient marker of gene expression in mammalian cells.

Keywords: *Renilla* luciferase; bioluminescence; coelenterazine; gene expression

INTRODUCTION

Light emission from the soft coral *Renilla reniformis* (Order Cnidaria) is catalyzed by a luciferase. Oxidation of the luciferin substrate, coelenterazine, by this luciferase leads to an excited state product (oxyluciferin) from which energy is transferred to the acceptor green fluorescent protein (GFP). GFP emits the green light ($\lambda_{max} = 509$) seen in the living coral (1). In the absence of GFP, luciferase-catalyzed oxidation of coelenterazine yields blue light ($\lambda_{max} = 480$ nm) (2).

A *Renilla* luciferase cDNA was cloned previously and expressed in *Escherichia coli* (3) and in plants (4). This cDNA was judged to be full length based on: (i) the deduced amino acid sequence; (ii) the apparent molecular size of the recombinant protein relative to native luciferase as determined from SDS-PAGE and Western blot analyses; and (iii) the catalytic and kinetic characteristics of the recombinant protein (3,5).

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The use of bioluminescence- and chemiluminescence-based reporter gene assays and immunoassays has increased dramatically in the past 10 years (6,7,8). Luciferases and photoproteins are especially useful since light emission can be measured and quantified easily, and they are non-toxic. A number of bacterial (9) and beetle (10,11) luciferase genes have been isolated and their uses have been well characterized. The bacterial *lux* genes have been used extensively as reporters, although the applications are primarily in procaryotic and plant hosts. *Lux* genes have a demonstrated utility as reporters of environmental pollutants and toxins, temporal and spatial developmental events, promoter strength and bacterial detection (see reviews 9,12,13). Similarly, the beetle luciferase *luc* genes have a well documented history as markers of transfection and transformation (14,15,16), as reporters of transcriptional regulation of genetic elements (17), and for determination of ATP concentrations *in vivo* and *in vitro* (18,19).

Other bioluminescence reporter genes include the *phot* genes which encode the calcium activated photoproteins aequorin (20) and obelin (21). The aequorin gene has been used to report Ca^{2+} flux in plants (22) and mammalian cells (23). Membrane-based assays using biotinylated recombinant aequorin have been developed for detection of nucleic and amino acids (24), and diagnostic immunoassays have been developed for a number of target molecules (25,26). The *luc* gene from the crustacean *Vargula hilgendorfii* has been isolated (27) and tested as a reporter gene (28); however, the substrate for this secreted luciferase is not readily available. Most recently, the gene encoding GFP from *Aequorea victoria* and has been used as a reporter of gene expression (29). It is not likely that any single reporter gene can offer the versatility required in the expanding number of applications that can be found for bioluminescence; each gene may have a particular advantage or disadvantage, depending on the assay and the conditions under which it is performed. Here, we report transient and stable expression of the *Renilla* luciferase r*luc* gene in COS-7 cells and C5 mouse fibroblasts, respectively, and demonstrate that this gene can be used both *in vitro* and *in vivo* as a marker gene in mammalian cells.

MATERIALS AND METHODS

Enzymes and media

Restriction enzymes, polymerases and ligases were obtained from New England Biolabs (Beverly, MA) and Promega (Madison, WI). PCR reagents and Taq polymerase were purchased from Perkin Elmer (Norwalk, CT). Media and chemicals required for the growth and maintenance of mammalian cell cultures were purchased from Gibco-BRL (Green Island, NY) and Sigma (St Louis, MO).

Modification of the luciferase cDNA

Plasmid pBSRLuc-1 was constructed by ligation of the 2.2 kbp SstI/EcoRI fragment isolated from pTZRLuc-1 (3) into plasmid Bluescript (Stratagene, La Jolla, CA) and transformed into *E. coli* DH5α cells. pBSRLuc-1 DNA was purified using a Qiagen tip-100 kit (Qiagen Inc, Chatsworth, CA) according to the manufacturer's instructions, linearized by digestion with SstI, and desalted in a G-25 spin column (Boheringer Mannheim Biochemicals) according to the manufacturer's instructions. Oligonucleotide primers were synthesized at the University of Georgia Molecular Genetics Sequencing Facility. The first oligo (GGCTGCAGATGACTTCGAAAGTTTAT) contained a PstI site immediately 5′ to the ATG start codon, followed by 18 nucleotides identical to the 5′ end coding sequence; the second (GGCTGCAGACATTTATATTATTAAACCC) also contained a PstI site and 20 nucleotides identical to the 3′ end immediately adjacent to an endogenous SmaI site located in the non-coding region. PCR amplification was performed using a programmable thermal cycler (MJ Research Inc, Watertown, MA). The PCR product was filled in with T-4 polymerase, isolated from a low melting temperature agarose gel (NuSeive; FMC, Rockland, ME) (30), and blunt-end ligated into alkaline phosphatase-treated SmaI digested pUC18 resulting in the plasmid pRLuc-4.1. *E. coli* DH5α cells were transformed and recombinant plasmid DNA was purified with a Qiagen tip-100. PstI and PstI–SmaI digests were performed to ensure correct orientation and size of the fragments.

Plasmid constructions

pRLuc-6 & pRLuc-6.1. Plasmid pRLuc-4.1 was digested with PstI and the insert was ligated into a unique PstI site contained on the mammalian expression vector pXM (31). Recombinants in pXM can be selected for by ampicillin resistance;

the luciferase gene is placed under the transcriptional control of the adenovirus major late promoter. *E. coli* DH5α cells were transformed and plasmid screens were performed to determine size and orientation of the insert DNA. Two clones, pRLuc-6.1 and pRLuc-6.1R, were isolated and contained the luciferase gene in the forward and reverse orientation, respectively, with respect to the promoter region.

pMCT-RUC. Plasmid pMCT-RUC (14 kbp) was constructed for site-specific targeting of the *Renilla* luciferase gene to a mammalian chromosome (details of the plasmid construction will be presented elsewhere). The relevant features of this plasmid are the *Renilla* luciferase gene under transcriptional control of the human cytomegalovirus immediate-early gene enhancer/promoter; the hygromycin gene under the transcriptional control of the thymidine kinase promoter; and a unique HpaI site is used to linearize the plasmid.

Mammalian cell culture

COS-7 cells. COS-7 cells were grown on 100 mm Costar plates as described previously (32). Cultures were trypsinized and split 1/20 2 days before use. Cell monolayers were 70–80% confluent at the time of transfection and were washed once in DME immediately prior to transfection. To each plate 3 mL DME was added containing 10 μg of plasmid DNA and 1.5 μL/μg Transfectam (Promega). After 5 h the transfection media was removed and the plates were washed once in DME. Fresh growth media was added to the cells, which were incubated for another 60 h. Cells were washed gently in PBS and then collected by scraping in 5 mL PBS/plate. Cells from one plate were centrifuged at 500 × *g* for 10 min, then resuspended in 0.5 mL 10 mmol/L Tris, 1 mmol/L EDTA, pH 7.6. Cells were incubated on ice for 10 min followed by sonication with a Branson Cell Disruptor using several 1 s bursts. Half of the cell extract was clarified by centrifugation at 15,000 × *g*, and luciferase activity was determined in the crude and clarified cell extracts.

C5 cells. C5 mouse fibroblasts were maintained as a monolayer as previously described (33). Cells at 50% confluency in 100 mm Petri dishes were used for calcium phosphate transfection (34) using 10 μg of linearized pMCT-RUC per plate. Colonies originating from single transfected cells were isolated and maintained in F-12 medium containing hygromycin (300 μg/mL) and 10% fetal bovine serum. Cells were grown in 100 mm Petri dishes prior to the *Renilla* luciferase assay.

Luciferase assays

COS-7 cells. The *Renilla* luciferase assay has been described previously (2). Assays of COS-7 cell extracts were performed in a luminometer (Turner model Td-20e) equipped with a sample chamber aperture designed to hold 12 × 75 mm tubes. The light path from the sample cell to the photomultiplier tube was restricted such that only the light passing through a 5 mm hole in base of the sample aperture was quantified. This design offered a geometry more suitable for reproducible measurements and calibration. The instrument was calibrated with a ^{14}C phosphor light standard for determination of quanta per second (35). For this instrument, 1 LU (light unit) = 6.4×10^6 hv/s. 10 μL of crude or clarified extracts were diluted into 1 mL luciferase assay buffer and injected rapidly into the sample tube containing 10 μL of 2.5 mmol/L coelenterazine in 1 mol/L HCl/MeOH. Integration time of the signal was 5 s.

C5 cells. Hygromycin-resistant cell lines obtained after transfection of mouse fibroblasts with linearized plasmid pMCT-RUC ("B" cell lines) were grown to 100% confluency for measurements of light emission both *in vivo* and *in vitro*. Light emission was measured *in vivo* after about 30 generations as follows: growth medium was removed and replaced by 1 mL RPMI 1640 containing coelenterazine (1 mmol/L final concentration). Light emission from cells was then visualized by placing the Petri dishes in a low light video image analyzer (Hamamatsu Argus-100). An image was formed after 5 min of photon accumulation using 100% sensitivity of the photon counting tube. For measuring light emission *in vitro*, cells were trypsinized and harvested from one Petri dish, pelleted, resuspended in 1 mL assay buffer (0.5 mol/L NACl, 1 mmol/L EDTA, 0.1 mol/L potassium phosphate, pH 7.4) and sonicated on ice for 10 s. Lysates were than assayed in a Turner TD-20e luminometer for 10 s after rapid injection of 0.5 mL of 1 mmol/L coelenterazine, and the average value of light

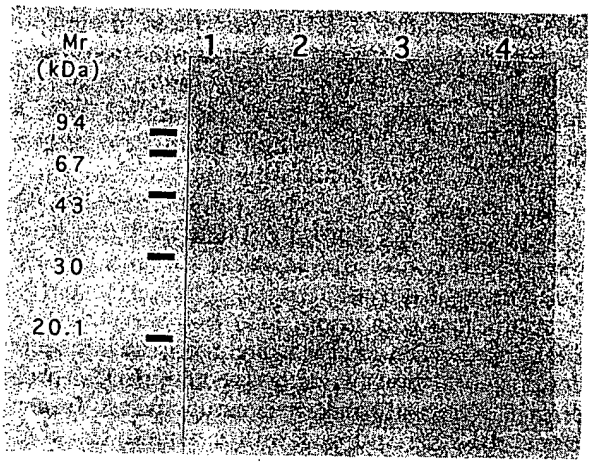


Figure 1. Western analysis of COS-7 cells transfected with the *Renilla* luciferase gene. Protein extract was fractionated with 12.5% SDS-PAGE before transfer to nitrocellulose as described in Methods. Lane 1, *Renilla* luciferase; Lane 2, pRLuc-6.1R transfected cells; Lane 3, pRLuc-6.1 transfected cells; Lane 4, pXM transfected cells

emission was recorded as LU (1 LU = 1.6×10^6 hν/s for this instrument).

SDS PAGE/Western blot analysis of recombinant luciferase

The protein concentration in lysates of cells expressing *Renilla* luciferase was determined by the method of Bradford (36). Clarified, crude extracts (50 μg) and native *Renilla* luciferase (0.5 μg) were fractionated on 12.5% SDS-PAGE gels (37). Western blot analysis was performed by transfer to nitrocellulose (Schleicher and Schnell, Keene, NH) (38). Luciferase detection was performed with a rabbit polyclonal anti-*Renilla* luciferase antibody (1/1000 dilution) and horseradish peroxidase conjugated goat anti-rabbit IgG second antibody (1/5000 dilution; Bio-Rad, Hercules, CA) as described previously (3).

RESULTS

COS-7 cells. Restriction analysis of the modified luciferase gene showed that: (i) it contained all the endogenous restriction sites of the template DNA as well as the additional PstI sites; and (ii) pRLuc-6.1 contained a single insert in the correct orientation with respect to the promoter and that pRLuc-6.1R contained a single insert in the reverse orientation (data not shown). An integrated value of 3708 LU was obtained for 10 μL of cell sonicate from pRLuc-6.1 transfected cells. This corresponds to approximately 2.3×10^{10} hν/s generated from 2% of the total protein released from the plate (Table 1). Sonicates clarified by centrifugation had a value of 3315 LU. No detectable light was measured from cells transfected with pXM or pRLuc-6.1R. It is important to note that the light intensity values reported above represent minimal ones, since the luciferase assays performed on these same samples with an unrestricted aperture (having a 25 mm bore) in the photometer sample chamber led to light emissions which exceeded the full scale limit of the instrument by a factor of 10^3 when at the lowest gain setting. Crude extracts from cells transfected with the above plasmids were analyzed by Western blotting (Fig. 1). A single protein band was seen in the pRLuc-6.1 transfected cell extracts (lane 3) but not in the pRLuc-6.1R (lane 2) or pXM (lane 4) cell extracts. Native *Renilla* luciferase (lane 1) is shown as a control. An identical blot incubated with preimmune serum failed to give a detectable signal (data not shown).

C5 cells. Independent cell lines of mouse fibroblasts transfected with linearized plasmid pMCT-RUC

Table 1. *In vitro* light emission from COS-7 cells transfected with plasmid pXM, pRLuc-6.1R, amd pRLuc-6.1. Values are an average of five measurements.

Plasmid	pXM	pRLuc-6.1R	pRLuc 6.1
L.U. (total crude extract)	0.0	0.0	3708 + / − 375
L.U. (clarified crude extract)	0.0	0.0	3315 + / − 540

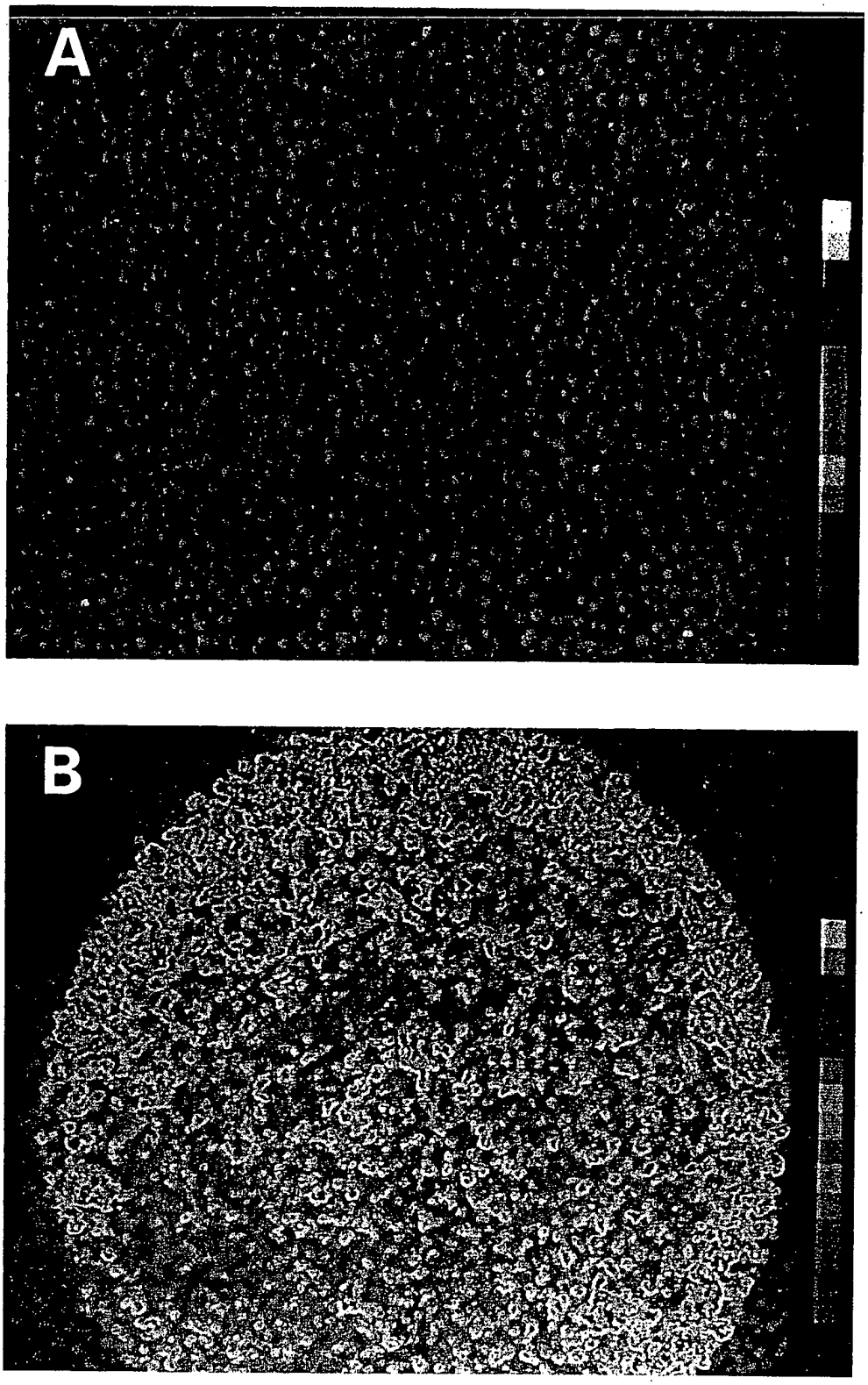
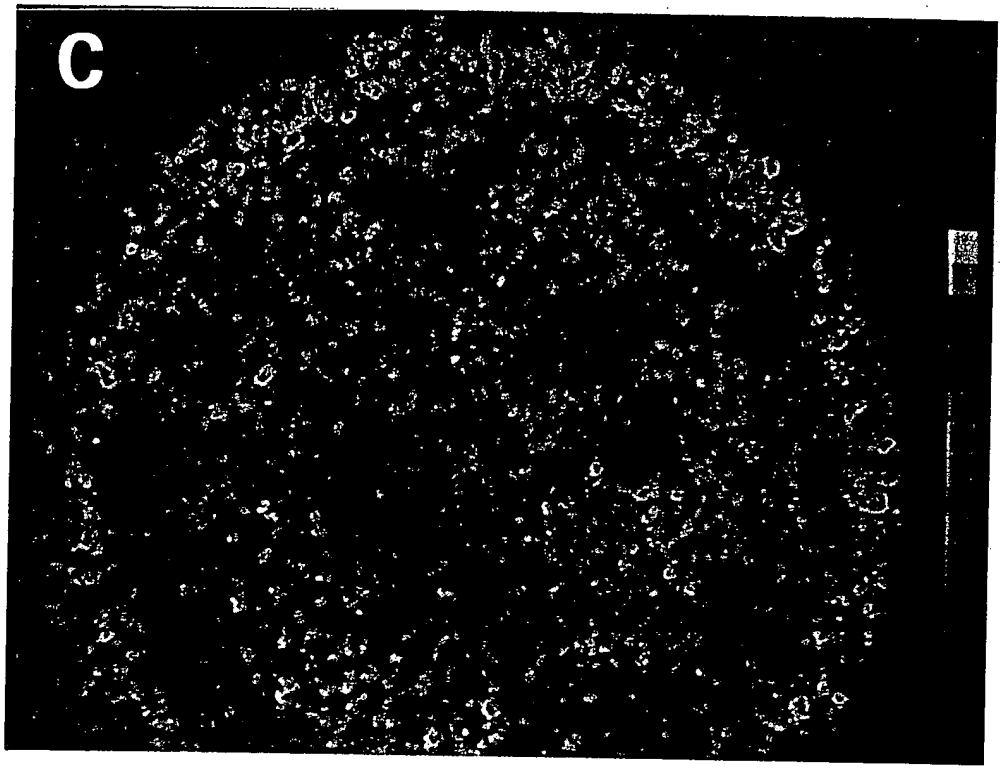


Plate 1. Low light video image analysis of Petri dishes (100mm) containing hyromycin-resistant mouse fibroblast cell lines ("B" cell lines) transfected with plasmid pMCT-RUC. a, cell line B3; b, cell line B6; c, cell line B9; d, cell line C5 (negative control)



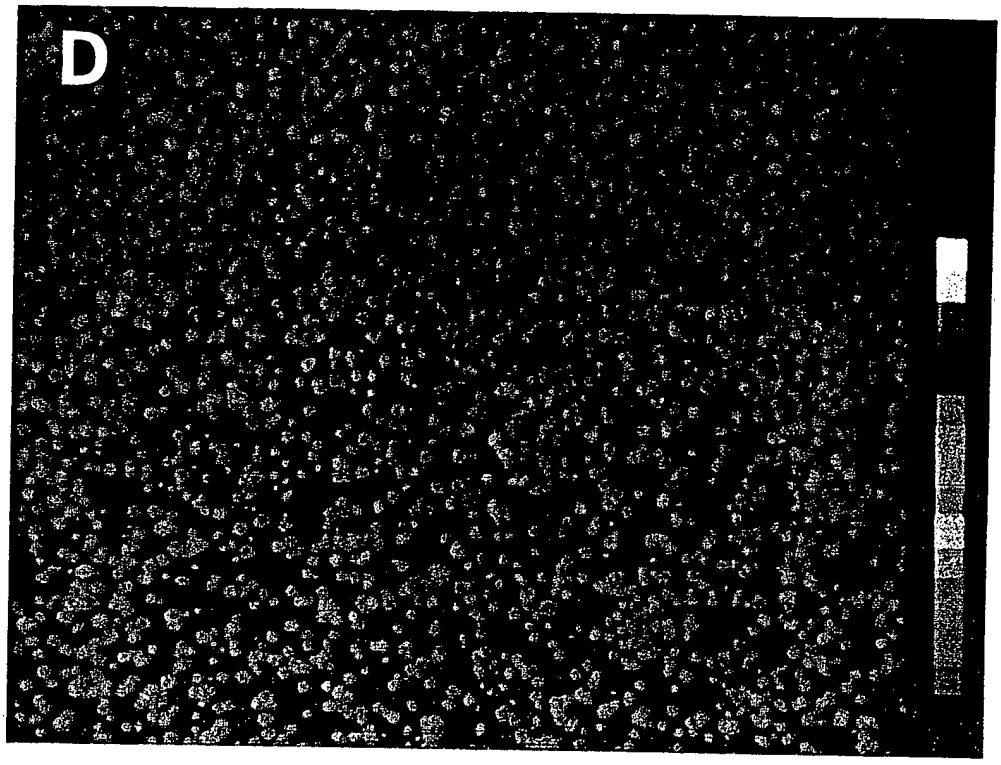


Plate 1. (cont.)

Table 2. *In vitro* light emission of C5 fibroblasts transfected with plasmid pMCT-RUC (B cell lines).

Cell Line	B3	B6	B9	C5
LU (clarified crude extract)	017.8	693.5	063.7	000.5

showed different levels of *Renilla* luciferase activity (Plate 1). Similar differences in light emission were observed when measurements were performed on lysates of the same cell lines (Table 2). This variation in light emission was probably due to a position effect resulting from the random integration of plasmid pMCT-RUC into the mouse genome, since enrichment for site targeting of the luciferase gene was not performed in this experiment.

DISCUSSION

The *Renilla* luciferase gene can be expressed and detected in a mammalian cell background. Transient expression in COS-7 cells showed that the *in vitro* bioluminescence emission from both total and clarified crude extracts exceeded 1×10^{10} hν/s. Also, the negligible difference between the light emissions from these two samples indicates that, as in expression in an *E. coli* host, recombinant *Renilla* luciferase exists as a soluble, cytoplasmic protein in transiently transfected mammalian cells. Analysis of these same extracts by Western blotting demonstrates that recombinant luciferase expressed in mammalian cells is essentially identical to the native protein, in both its molecular size and immunological reactivity. In addition, the stable expression of the *Renilla* luciferase gene in C5 fibroblasts demonstrates non-toxicity of the gene product in mammalian cells, and confirms that coelenterazine will readily permeate mammalian cell membranes (23). Unlike photoproteins, *Renilla* luciferase requires no time period for charging an apoprotein prior to assay and signal detection. The ease of detection of luciferase activity in transiently transfected cells makes the *Renilla* luciferase gene an ideal candidate as a marker of transfection, as well as a reporter gene of genetic events associated with transcription and translation.

Renilla luciferase requires only O_2 and the substrate coelenterazine, which is commercially available. The fact that the *Renilla* luciferase gene has been expressed at high levels in bacterial, plant and animal cells in a stable and transient fashion demonstrates its utility and versatility in the field of bioluminescence-based detection. Further testing of *Renilla* luciferase vectors may reveal that it is superior to other luciferases when used in some applications, because it requires no divalent cations as does aequorin, no ATP as do the beetle luciferases, and no long-chain aldehydes as do the bacterial luciferases. Also, mammalian membrane permeability to coelenterazine does not appear to pose a problem.

Assay and detection methods based on currently available bioluminescence genes offer sensitive and reliable alternatives to other isotopic and non-isotopic methods. There are, however, inherent problems, such as temperature instability (39,40), low turnover (41,42), rigid ionic strength/buffer constraints (43,44,45), lack of commercial availability of substrate (i.e. *Vargula* luciferin), ATP dependency (4,44), and susceptibility to proteolysis (46,47) which limit the usefulness of currently available luciferases and photoproteins. Preliminary evidence suggests that the expressed *Renilla* luciferase is stable with respect to elevated temperatures and a wide range of ionic strengths (5). Finally, when a dual marker system is needed, it should be noted that the *Renilla* luciferase gene used in conjunction with the firefly gene may fill this need, since the large difference in their peak light emissions (480 nm (2) and 562 nm (48), respectively) could be measured simultaneously.

REFERENCES

1. Cormier MJ. Comparative Biochemistry of Animal Systems. In: Herring PJ, editor. Bioluminescence in action. New York: Academic Press, 1978:75–108
2. Matthews JC, Hori K, Cormier MJ. Purification and properties of *Renilla reniformis luciferase*. Biochemistry 1977;16:85–91.
3. Lorenz WW, McCann RO, Longiaru M, Cormier MJ. Isolation and expression of a cDNA encoding *Renilla* luciferase. Proc Natl Acad Sci USA 1991;88:4438–2.
4. Mayerhofer R, Langridge WHR, Cormier MJ, Szalay AA. Expression of recombinant *Renilla* luciferase in transgenic plants results in high levels of light emission. Plant J 1995;7:1031–1038.

5. Lorenz WW, Gray JP, Cormier MJ, Gibson BG, O'Kane DJ. Overexpression and purification of recombinant *Renilla reniformis* luciferase. In: Szalay A, Kricka LJ, Stanley PE, editors. Bioluminescence and chemiluminescence: status report. Chichester: Wiley, 1993; 191–5.
6. Kricka LJ. Selected strategies for improving sensitivity and reliability of immunoassays. Clin Chem 1994;40:347–57.
7. Bronstein I, Fortin J, Stanley PE, Stewart GSAB, Kricka LJ. Chemiluminescent and bioluminescent reporter gene assays. Anal Biochem 1994;219:169–81.
8. Bronstein I, Fortin JJ, Voyta JC, Jou RR, Edwards B, Olesen CEM et al. Chemiluminescent reporter gene assays: sensitive detection of the GUS and SEAP gene products. BioTechniques 1994;17:172.
9. Meighen EA. Bacterial bioluminescence: organization, regulation, and application of the *lux* genes. FASEB J 1993;7:1016–22.
10. Wood KV, Lam YA, McElroy WD. Introduction to beetle luciferases and their applications. J Biolumin Chemilumin 1989;4:289–301.
11. Devine JH, Kutozova GD, Green VA, Ugarova NN, Baldwin TO. Luciferase from the last european firefly *Luciola mingrelica*—cloning and nucleotide sequence of the cDNA, overexpression in *E. coli* and purification of the enzyme. Biochem Biophys Acta 1993;1173:121–32.
12. Barker JM, Griffiths MW, Collins-Thompson DL. Bacterial bioluminescence: applications in food microbiology. J Food Protection 1992;55:62–70.
13. Stewart GSAB, Williams P. *lux* genes and the applications of bacterial bioluminescence. J Gen Microbiol 1992;138:1289–1300.
14. Jiang C, Langridge WHR, Szalay AA. Identification of plant genes *in vivo* by tagging with EDNA border-linked luciferase genes followed by inverse polymerase chain reaction amplification. Plant Mol Biol Reporter 1992; 10:345–61.
15. Gelmini S, Pinzani P, Orlando C, Sestini R, Baldwin TO & Pazzagli M. Chloramphenicol acetyl-transferase, firefly and bacterial luciferases as reporter genes in transfection of mammalian cells. In: Szalay AA, Kricka LJ, Stanley P, editors. Bioluminescence and chemiluminescence. Chichester: Wiley, 1993;200–6.
16. Vieites JM, Navarro-Garcia F, Perez-Diaz R, Pla J, Nombela C. Expression and *in vivo* determination of firefly luciferase as a reporter in *Saccharomyces cerevisiae*. Yeast 1994;10: 1321–7.
17. Lira SA, Kinloch RA, Mortillo S, Wasserman PM. An upstream region of the mouse ZP3 gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. Proc Natl Acad Sci USA 1990;87:7215–9.
18. Koop A, Cobbold PH. Continuous bioluminescent monitoring of cytoplasmic ATP in single isolated rat hepatocytes during metabolic poisoning. Biochem J 1993;295:165–70.
19. Seeger K, Griffiths MW. Adenosine triphosphate bioluminescence for hygiene monitoring in health care institutions. J Food Protection 1994;57:509–12.
20. Prasher D, McCann RM, Cormier MJ. Cloning and expression of the cDNA coding for aequorin, a bioluminescent calcium-binding protein. Biochem Biophys Res Commun 1985;126:1259–68.
21. Zakharchenko VA, Gitelson GI. Gene of photoprotein obelin from *Obelia longissima* as a reporter gene. Bioluminescence Symposium, Maui, 1993.
22. Knight MR, Campbell AK, Smith SM, Trewavas AJ. Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. Nature 1991;352: 524–6.
23. Button D, Brownstein M. Aequorin-expressing mammalian cell lines used to report Ca^{2+} mobilization. Cell Calcium 1993;14:663–71.
24. Stults NL, Stocks NF, Rivera H, Gray J, McCann RO, O'Kane DO et al. Use of recombinant biotinylated aequorin in microtiter and membrane-based assays: purification of recombinant apoaequorin from *Escherichia coli*. Biochemistry 1992;31:1433–42.
25. Rigel CT, Patel MT, Rivera HN, Stults NL, Smith DF. A bioluminescent assay for testosterone based on the recombinant photoprotein, aequorin. In: Campbell AK, Kricka LJ, Stanley PE, editors. Bioluminescence and chemiluminescence. Fundamentals and applied aspects. Chichester: Wiley, 1994:345–8.
26. Rivera HN, Patel MT, Stults NL, Smith DF, Rigel CT. A single addition interleukin-6 bioluminescent immunoassay features recombinant aequorin as a dried reagent. In: Campbell AK, Kricka LJ, Stanley PE, editors. Bioluminescence and chemiluminescence.

Fundamentals and applied aspects. Chichester: Wiley, 1994:203–6.
27. Thompson EM, Nagata S, Tsuji FI. Cloning and expression of cDNA for the luciferase from the marine ostracod *Vargula hilgendorfii*. Proc Natl Acad Sci USA 1989;86: 6567–71.
28. Thompson EM, Nagata S, Tsuji FI. *Vargula hilgendorfii* luciferase: a secreted reporter enzyme for monitoring gene expression in mammalian cells. Gene 1990;96:257–62.
29. Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994; 263:802–5.
30. Weislander L. A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. Anal Biochem 1979;98:305–9.
31. Kaufman RJ. Identification of the components necessary for adenovirus translational control and their utilization in cDNA expression vectors. Proc Natl Acad Sci USA 1985;82:689–93.
32. Gluzman Y. SV40-transformed simian cells support the replication of early SV40 mutants. Cell 1981;23:175–82.
33. Hadlaczky G, Praznovsky T, Cserpan I, Kereso J, Peterfy M, Kelemen I et al. Centromere formation in mouse cells cotransformed with human DNA and a dominant marker gene. Proc Natl Acad Sci USA 1991;88:8106–10.
34. Harper ME, Saunders GF. Localization of single copy DNA sequences on G-banded human chromosomes by *in situ* hybridization. Chromosoma 1981;83:431–9.
35. O'Kane DJ, Lee J. Encapsulated radiophosphorescent standards for day-to-day photometer calibration. Photochem Photobiol 1990; 52:723–34.
36. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of proteins utilizing the principle of protein-dye binding. Anal Biochem 1976;72:248–54.
37. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;277:680–5.
38. Burnett WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radio-iodinated protein A. Anal Biochem 1981;112:195–203.
39. Escher A, O'Kane DJ, Lee J, Szalay AA. Bacterial luciferase alpha beta fusion protein is fully active as a monomer and highly sensitive *in vivo* to elevated temperature. Proc Natl Acad Sci USA 1989;86:6528–32.
40. Escher A, O'Kane DJ, Szalay AA. The beta subunit polypeptide of *Vibrio harveyi* luciferase determines light emission at 42°C. Mol Gen Genetics 1991;230:385–93.
41. Tanahashi H, Ito T, Inouye S, Tsuji FI, Sakaki Y. Photoprotein aequorin: use as a reporter enzyme in studying gene expression in mammalian cells. Gene 1990;96:249–55.
42. Li Z, Meighen EA. The turnover of bacterial luciferase is limited by a slow decomposition of the ternary enzyme-product complex of luciferase, FMN, and fatty acid. J Biol Chem 1994;269:6640–4.
43. Pazzagli M, Devine JH, Peterson DO, Baldwin TO. Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. Anal Biochem 1992;204:315–23.
44. Chittock RS, Lidzey DG, Berovic N, Wharton CW, Jackson JB, Beynon TD. The quantum yield of luciferase is dependent on ATP and enzyme concentrations. Mol Crystals Liq Crystals 1993;234:599–604.
45. Kajiyama N, Nakano E. Thermostabilization of firefly luciferase by a single amino acid substitution at position 217. Biochemistry 1993; 32:13795–9.
46. Holzman TF, Baldwin TO. Proteolytic inactivation of luciferases from three species of luminous marine bacteria, *Beneckea harveyi*, *Photobacterium fisheri*, and *Photobacterium phosphoreum*: evidence of a conserved structural feature. Proc Natl Acad Sci USA 1980;77:6363–7.
47. Thompson JF, Hayes LS, Lloyd DB. Modulation of firefly luciferase stability and impact on studies of gene regulation. Gene 1991; 103:171–7.
48. McElroy WD, Seliger HH. Mechanism of action of firefly luciferase. Fed Proc 1962; 21:1006–12.